Molecular Characterization of Local Isolates of *Mycoplasma capricolum* Sub Specie *Capripneumoniae* in Goats (*Capra hircus*) of Khyber Pakhtunkhwa, Pakistan

Muhammad Kamal Shah1*, Umer Saddique1, Shakoor Ahmad1, Aqib Iqbal2, Abid Ali2, Waseem Shahzad3, Muhammad Sayyar Khan2, Hamayun Khan1, Haifur Rahman1, Said Sajjad Ali Shah1 and Muhammad Israr4

1Department of Animal Health, Faculty of Animal Husbandry & Veterinary Sciences, the University of Agriculture, Peshawar, Pakistan; 2Institute of Biotechnology & Genetic Engineering, University of Agriculture, Peshawar; 3Veterinary Research Institute, Lahore, Pakistan; 4Pakistan Science Foundation, Islamabad, Pakistan

*Corresponding author: kamaluaf560@gmail.com*

**ARTICLE HISTORY**

Received: August 13, 2016

Revised: September 21, 2016

Accepted: October 30, 2016

Published online: November 23, 2016

**Key words:**

Goat

Hay Flick media

Mycoplasma

Pakistan

PCR

Sequencing

**A B S T R A C T**

Caprine mycoplasmosis is an important infectious respiratory complication lead to significant health issue and causes heavy economic losses in small ruminant population throughout the world. The study was designed to identify and characterize the pathogenic member of mycoplasma cluster the *Mycoplasma capricolum* sub specie *capripneumoniae* (*Mccp*) isolated from goat suffering from respiratory syndrome in the natural outbreak. The study was carried out during November, 2013 to April, 2015 in the Khyber Pakhtunkhwa (KPK), Pakistan. A total of 825 samples from nasal discharge, tracheal swab, pleural fluid and lung tissue were collected from goat clinically suspected for Contagious Caprine Pleuro Pneumonia (CCPP). The samples taken in PPLO transport media were cultured on modified Hay Flick media and incubated at 37°C with 5% CO2 for 7-12 days. Out of total 267 (32.36%) were positive for mycoplasma growth showing mass turbidity, whirling movement in culture broth and typical fried egg colonies in agar media. The positive culture was identified through biochemical assay and confirmed as *Mccp* through PCR by using cluster and specie specific primers. Out of 267 positive samples 55 (20.59%) were confirmed as *Mycoplasma mycoides* cluster and 23 (8.61%) were identified as *Mccp*. Sequencing of the 16-S rRNA gene blast search revealed 99% sequence homology with *Mccp* compared with eight available sequences at NCBI. The presence of *Mccp* was for the first time reported in small ruminants of Khyber Pakhtunkhwa.

©2016 PVJ. All rights reserved


**INTRODUCTION**

Mycoplasmosis is the most important respiratory disease poses a serious threat to the small ruminant population by causing heavy economic losses in Northern and Southern regions of the country (Saddique et al., 2012; Samiullah, 2013; Banaras et al., 2016). Among the different pathogenic species of mycoplasma, contagious caprine pleuropneumonia (CCPP) is highly fatal disease caused by *Mycoplasma capricolum* sub specie *capripneumoniae* (*Mccp*) (OIE, 2012). The CCPP was first time reported in Algeria in 1873 and later in many countries of Africa, Europe, Middle East and Asia (Tigga et al., 2014; Atim et al., 2016). In Pakistan the disease was considered to be caused by *Mycoplasma mycoides* sub specie *capri* till the molecular confirmation as *Mccp* in Baluchistan by Awan et al. (2010). Recently in the international collaborative study *Mccp* was confirmed in the northern Pakistan and Tajikistan (Peyraud et al., 2014). The disease also prevalent in different areas and livestock research centers of Punjab, Pakistan (Shahzad et al., 2016). The disease is widely spread in many countries of the globe and considered to be caused by six different pathogenic species called as *Mycoplasma* cluster. However, the classical form of disease is caused by *Mycoplasma capricolum* sub specie *capripneumoniae* that mainly confined to the thoracic cavity (Thiaucourt and Boksle, 1996; Manzo-Silvan et al., 2007).

The classical signs of CCPP are characterized by respiratory distress, high fever (41-43°C), high morbidity and mortality in susceptible herds irrespective of age and sex. There is dyspnea accompanied by grunting and
The disease mainly confined to the thoracic cavity characterized by unilateral sero-fibrinous pleuropernemonia with severe pleural effusion and hepatization (Mondal et al., 2004). In some acute cases the pleural cavity contains an excessive straw colored fluid with fibrin flocculations (Sadique et al., 2012). In per acute cases, minimal clinical signs are noted with mortality within 1-3 days (Samiullah, 2013). Histopathological examination of affected lungs shows sero-fibrinous necrotic pneumonia, polymorphic nuclear neutrophil infiltration in alveolar spaces. The susceptible species of animals are domestic sheep (Ovis aries), goat (Capra hircus) and wild ruminants including wild goats (Capra aegagrus), Gerenuk (Litocranius walleri), Nubian Ibex (Capra ibex nubiana) and Lasrtian mouflon (Ovis orientalis lasistanaica). It causes significant losses in these animals with high morbidity and mortality (Arif et al., 2007).

A lot of work has been carried out for the diagnosis and identification of mycoplasma species by using different conventional techniques in Pakistan. But little work has been conducted on molecular characterization of the local isolates of mycoplasma species. Mostly the conventional methods of identification failed to address the issue properly because of its shortcoming. The isolation of mycoplasma is difficult due to its fastidious nature and needs special media and proper conditions for growth (OIE, 2012). The serological and biochemical tests are usually fail due to sharing of common antigenic epitopes by many species of mycoplasma. Therefore, the advanced molecular techniques like PCR and sequencing is the most accurate tool for identification and confirmation of different mycoplasma species (Woubit et al., 2004). It can confirm the exact specie of microorganism even in mixed infection and directly from clinical samples like nasal discharge and pleural fluids. The mycoplasma having 16S rRNA gene allowed the identification of variable regions with both genus and species specific primers to identify the particular species of mycoplasma cluster (Hotzel et al., 1996; Manso-Silvan et al., 2007; Kumar et al., 2011). A new technique the Recombinase Polymerase Amplification Assay is developed for the rapid and accurate detection of different strains of Mccp in Kenya. However, this technique is costly and not easily accessible in the developing countries (Liljander et al., 2015). Looking at the paucity of the scientific literature on mycoplasma in Pakistan the present work is carried out to identify, characterize and recognize the never reported Mycoplasma capripneumoniae sub specie capripneumoniae in small ruminants of Khyber Pakhtunkhwa. This study will have paved a way for researcher and planner to design strategies for curbing this fatal disease.

MATERIALS AND METHODS

Sampling: A total of 825 samples consisted of nasal, tracheal, pleural fluid and lung tissue were collected from goats exhibiting the signs of respiratory distress suspected for CCPP in Khyber Pakhtunkhwa Pakistan. The samples were taken by sterile swab and then transfer to the transport media. The collected samples were kept under refrigeration and transported to the Pathobiology Lab Department of Animal Health, the University of Agriculture, Peshawar, Pakistan for onward processing.

Isolation and identification: The samples were taken aseptically by sterile swab and transferred to the PPLO broth as a transport media. All the collected samples were incubated in anaerobic incubator (New Brunswick, Galaxy 48-S UK) with 5% CO2 at 37°C for 5-10 days. The test tubes were examined daily for presence of mass turbidity, whirling movement and change in color with decreased in pH. The positive growths were cultured on Hay Flick agar for the appearance of nipple like or fried egg typical mycoplasma colonies. The positive colonies were re-cultured three time for obtaining pure culture as per standard protocol of (OIE, 2012).

Biochemical assay: Biochemical assay of the local isolates was carried out for identification of the specie of the mycoplasma cluster as per standard protocol of (Adehan et al., 2006). A volume of 0.5µl from each isolate were diluted in 5ml of Hay Flick broth and subjected to different biochemical tests like glucose fermentation, serum digestion, Tetrazolium reduction (aerobically and anaerobically), casein digestion and arginine hydrolysis test for the identification of desired mycoplasma species.

Polymerase chain reaction: The biochemical identified samples of the species of mycoplasma Mccp were subjected to DNA extraction for confirmation through PCR. The polymerase chain reaction (PCR) was performed for the detection of mycoplasma species by using two set of primers the Mycoplasma mycoides cluster and specie specific as described by Hotzel et al. (1996). These primers targeted the 16S rRNA gene of mycoplasma with an amplicon size of 548 and 316 bp for Mycoplasma mycoides cluster and Mycoplasma capricolum sub specie capripneumoniae respectively (Table 1).

Homology and Phylogenetic analysis: The gel product of specific amplicon size was taken and submitted for sequencing. The obtained sequence was subjected to NCBI BLAST to screen for homologous sequences for phylogenetic relation of the local isolates of Mycoplasma capricolum sub specie capripneumoniae with available sequences. Sequences of the isolates were downloaded from NCBI and were multiple aligned through BioEdit version 7.0.5.2 (Hall, 1999). Furthermore, Phylogenetic tree topology was constructed for the obtained sequences using software MEGA version 7.1 for evolutionary study and to build correlation with other strains of different regions (Tamura et al., 2011).

<table>
<thead>
<tr>
<th>Mycoplasma species</th>
<th>Primer designation</th>
<th>Oligonucleotide sequence 5’-3’</th>
<th>Annealing Tm (C)</th>
<th>Expected amplicon size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mycoides cluster</td>
<td>Mm-F</td>
<td>(CCA AAG GGC CTT ACT GGC TTT TT)</td>
<td>52</td>
<td>548</td>
<td>Hotzel et al., 1996</td>
</tr>
<tr>
<td></td>
<td>Mm-R</td>
<td>(TTG AGA TTA GCT CCC CTT CAC AG)</td>
<td>56</td>
<td>54</td>
<td>Woubit et al., 2004</td>
</tr>
<tr>
<td>Mccp</td>
<td>Mccp.spe-F</td>
<td>(ATC ATT TTT AAT CCC TTC AAG)</td>
<td>54</td>
<td>316</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mccp.spe-R</td>
<td>(TAC TAT GAG TTA TTA TAT ATG CAA)</td>
<td>54</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Statistical analysis: Data was compiled in Microsoft Excel and analyzed through Chi-square test using SPSS 19.0 SOFTWARE to check statistical difference between different sources of samples.

RESULTS

Isolation of mycoplasma: Out of 825 samples 267 (32.36%) were positive on culture for mycoplasma showing mass turbidity and change in color in PPLO broth media. A typical nipple like and fried egg colonies were appeared on day 4th and 7th post incubation in Hay Flick agar media (Fig. 1 & 2). Highest culture was obtained from pleural fluid followed by lung tissue, tracheal and nasal discharge. However, on statistical analysis of the data by Chi-square test ($\chi^2$) non-significant association (P>0.05) was found between the four different sources of samples obtained from goats suspected for CCPP. The results of growth of *Mycoplasma capricolum* sub specie capripneumoniae on culture media are presented in the (Table 2).

The positive culture of mycoplasma was sub cultured on modified Hay Flick agar till the characteristic typical nipple like and fried egg colonies were obtained. On biochemical analysis 143 (17.33%) was positive for glucose fermentation, serum digestion, tetrazolium reduction test and casein digestion test while negative for arginine hydrolysis test (Table 4).

Molecular characterization: Based on PCR analysis, out of total 267 cultured samples 55(20.59%) were identified as *Mycoplasma mycoides* cluster with an amplicon size of 548 bp. On analysis of data ($\chi^2$) significant (P<0.001) result was obtained from pleural fluids (43.33%) followed by lungs tissue (27.14%) among the sources of samples (Table 3). Out of 55 PCR confirmed mycoides cluster, 23(8.61%) were positive for Mccp with an amplicon size of 316 bp (Fig. 3 & 4). The *Mycoplasma capricolum* sub specie capripneumoniae was identified for the first time in small ruminants of Khyber Pakhtunkhwa, Pakistan. Ten PCR confirmed local isolates were processed for sequencing and the sequence of the PCR product obtained through specie specific primers showed maximum sequence homology 99% of 16-S rRNA gene of *Mycoplasma capricolum* sub specie capripneumoniae with the strains of neighbor countries. The phylogenetic tree was constructed by using software Mega version 7.0.5.2 and compared with 08 available sequences in NCBI gene data bank. The constructed tree indicate that the local isolated field strain is different from the strains of USA and France but closely related with the strain of neighbor countries like India and China (Fig. 5).

<table>
<thead>
<tr>
<th>Nature of sample</th>
<th>Culture Media</th>
<th>Pearson Chi-square</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nasal Swab</td>
<td>151 (30.2)</td>
<td>349 (69.8)</td>
<td>500</td>
</tr>
<tr>
<td>Tracheal swab</td>
<td>74 (32.9)</td>
<td>151 (67.1)</td>
<td>225</td>
</tr>
<tr>
<td>Lung Tissue</td>
<td>28 (40)</td>
<td>42 (60)</td>
<td>70</td>
</tr>
<tr>
<td>Pleural fluid</td>
<td>14 (46.7)</td>
<td>16 (53.3)</td>
<td>30</td>
</tr>
<tr>
<td>Total</td>
<td>267 (32.4)</td>
<td>558 (67.6)</td>
<td>825</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>M</th>
<th>C+ 1 2 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>1000bp</td>
<td></td>
</tr>
<tr>
<td>500bp</td>
<td></td>
</tr>
<tr>
<td>316bp</td>
<td></td>
</tr>
<tr>
<td>250bp</td>
<td></td>
</tr>
</tbody>
</table>

Table 2: Results of culture of mycoplasma obtained from different sources of samples on modified Hay Flick media collected from goats suffering from respiratory diseases suspected for CCPP. Statistical analysis of the data showed non-significant association (P>0.05) between the different sources of samples.

Fig. 1: Typical *Mycoplasma capricolum* sub sp. capripneumoniae colony with fried egg appearance obtained on day 7th post incubation on modified Hay Flick agar, isolated from lungs tissue of naturally infected goat at 10X.

Fig. 2: *Mycoplasma capricolum* sub sp. capripneumoniae colonies with nipple like appearance on day 4th post incubation on modified Hay Flick agar, isolated from the pleural fluid of naturally infected goats at 4X.

Fig. 3: PCR product of *Mycoplasma capricolum* subsp. capripneumoniae amplicon size 316 bp in samples collected from goats exhibiting the sign CCPP. M=1kb DNA ladder, Samples = 1, 2, 3, C+ = Positive control.

Fig. 4: PCR product of *Mycoplasma mycoides* cluster with an amplicon size of 548 bp in samples collected from goat exhibiting the sign of CCPP. M = 1KB DNA ladder, Sample = 1, 2, 3, N = Negative Control, C+ = Positive Control.
Table 3: PCR based identification of Mycoplasma mycoides cluster from different clinical samples of goats suspected for CCPP in Khyber Pakhtunkhwa, Pakistan. Statistical analysis (χ²) showed significant association (P<0.001) between the isolates and different sources of samples.

<table>
<thead>
<tr>
<th>Status</th>
<th>Nasal (%)</th>
<th>Tracheal (%)</th>
<th>Lungs tissue (%)</th>
<th>Pleural fluids (%)</th>
<th>Total (%)</th>
<th>Chi-square</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>17 (3.4)</td>
<td>06 (2.66)</td>
<td>19 (7.24)</td>
<td>13 (4.33)</td>
<td>55 (6.66)</td>
<td>126.35</td>
<td>0.001</td>
</tr>
<tr>
<td>Negative</td>
<td>483 (96.6)</td>
<td>219 (97.33)</td>
<td>51 (72.83)</td>
<td>17 (56.66)</td>
<td>770 (93.33)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>500</td>
<td>225</td>
<td>70</td>
<td>30</td>
<td>825</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Fig. 5:** Phylogenetic relationship of the Mycoplasma capricolum sub specie capripneumoniae sequence obtained (KPK, Pakistan) comparing with other eight isolates available sequences in NCBI. Sequences of the isolates were downloaded from NCBI and were aligned through BioEdit multiple alignment. The phylogenetic tree was constructed by neighbor-joining algorithm using the software MEGA version 7.0.5.2.

Table 4: Results of different biochemical assay on culture of mycoplasma isolated from samples collected from goat suffering from respiratory syndrome for the confirmation of mycoplasma cluster and Mcp.

<table>
<thead>
<tr>
<th>S/No</th>
<th>Biochemical assay</th>
<th>Sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Glucose fermentation</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>Serum digestion</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>Tetrázolium reduction</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>Casein digestion</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>Arginine hydrolysis</td>
<td>-</td>
</tr>
</tbody>
</table>

+ Positive, - Negative

**DISCUSSION**

Mycoplasmosis is important respiratory disease of small ruminant, causing heavy economic losses throughout the country, especially in Northern and Southern regions of Pakistan (Saddique et al., 2012; Hira et al., 2015; Banaras et al., 2016). Different pathogenic species are reported throughout the country, several conventional and non-conventional techniques are used for the identification of different pathogenic species of mycoplasma with various degree of success. The presence of Mcp and other cluster specie was investigated and successfully isolated from nasal, tracheal, lungs tissue and pleural fluid. The member of Mycoplasma mycoides cluster in small ruminants has wide range of tissue tropism and causes infections in various body organs. The Mcp is the ability to cause acute respiratory infection which mostly confined to the thoracic cavity (OIE, 2012). Maximum growth of Mcp was obtained from pleural fluid followed by lungs tissue, tracheal and nasal discharge of goats in the study area. Similar observations were also made by (Nicholas et al., 2002; Sadique et al., 2012; Liljander et al., 2015). It is revealed from the present findings that most desirable source of samples are lungs tissue and pleural fluids for the successful isolation of Mcp. The isolation of Mcp has also been reported from tracheal and nasal discharge (Kumar et al., 2011; Zinka et al., 2013). It is justified by the fact that as the disease progressed the purulent pulmonary discharge come through upper respiratory tract along with cough which contain the microorganism. The isolates were successfully grown on modified Hay Flick media from different clinical specimen of the infected animals. Apart from other member of mycoplasma cluster the Mcp has the characteristic to grow slowly on modified Hay Flick agar media. However, a typical fried egg and nipple like colonies were observed on modified Hay Flick agar medium on day 7th and 9th during first inoculation, while the same colonies were observed on day 4-7th in third passage. Similar observations were also reported by Hernandez et al. (2006).

Both conventional and non-conventional techniques are used for diagnosis of CCPP with varying degree of success. Mostly the serological and biochemical techniques are failed due to sharing of antigenic epitopes of Mycoplasma mycoides clusters (Nicholas et al., 2002). The biochemical assay is still used to differentiate the pathogenic species of mycoplasma in developing countries (OIE, 2012). The digitonin sensitivity distinguishes Mycoplasma from Acholeplasma, serum digestion distinguishes Mycoplasma cluster members from all other species of mycoplasma of small ruminant (Samullah, 2013). All the local isolates were positive for glucose fermentation, serum digestion, tetrázolium reduction test and casein digestion test while negative for arginine hydrolysis. The results are supported by the finding of (Nicholas et al., 2002). It is further justified by the findings that the Mcp lack the ability to digest arginine which differentiate it from Mycoplasma capricolum sub specie capricolum (Noah et al., 2011). In Pakistan the CCPP is consider to be caused by Mycoplasma mycoides subspecies capri by using conventional methods (Rehman et al., 2003). Later on molecular identification of the same specie was reported Shahzad et al. (2012) in Punjab, Awan et al. (2012); Hira et al. (2015) in Baluchistan and Sadique et al. (2012) in Khyber Pakhtunkhwa. However, the causative agent Mcp was for the first time isolated and confirmed through molecular diagnosis in district Pishin, Baluchistan Pakistan by Awan et al. (2010). The molecular detection has greatly improved CCPP diagnosis even in mixed culture and also directly from clinical specimen like nasal discharge and pleural fluids. PCR based on 16-S rRNA gene analysis allow the detection of Mycoplasma mycoides cluster and other species with accurate identification (Manos-Silvan et al., 2007; Kumar et al., 2011).

In the northern Pakistan the Mycoplasma capricolum sub specie capripneumoniae (Mcpp) was for the first time isolated and confirmed through PCR by using species specific primers. Out of total 55 PCR confirmed mycoplasma cluster 23(8.61%) were positive for Mcpp with an amplicon size of 316 bp. The study agrees with the findings of the prevalence of Mcp in Baluchistan, Pakistan by Awan et al. (2010). It is further supported by the findings that the seroprevalence of CCPP caused by Mcp was 2.7% and 44.2% in Gilgit and Diamer Districts of Northern Pakistan and 10.1% in the Shuro-Odob District of Tajikistan (Peyraud et al., 2014). Similar findings were
also reported in different areas of Punjab, Pakistan reflecting the seroprevalence of Mccp 8.52% (Shahzad et al., 2016). It is reported that CCPV is prevalent in 40 countries but Mccp has been isolated only in 17 countries (Manso-Silvan et al., 2011). However, now it has been reported that Mccp is prevalent in many country of the world including China and Tajikistan (Chu et al., 2011). The Northern areas of Pakistan are adjacent with Afghanistan and Tajikistan hosted a large population of sheep and goat throughout the year. This influx of small ruminants from these neighbor countries is the major risk factor of cross boundary infectious diseases of livestock particularly the CCCP. In Pakistan only one specie specific vaccine (Mycoplasma mycoides sub specie capri) is available and used as prophylactic measures for the control of this disease. The failure of vaccine justifies the prevalence of disease caused by Mccp as the different specie of mycoplasma having no cross immunity for protection. The sequence study of Mccp revealed that the local isolates showed close relation with isolates of China and India, but having evolutionary distance from strains of other countries like USA, France and Switzerland.

Conclusions: It is concluded that Mycoplasma capricolum sub specie capripneumoniae is wide spread pathogenic specie isolated from goat population in natural outbreak throughout Khyber PakhtunKhwa, Pakistan. The isolated specie of Mccp having close homology with the strains of neighboring countries like China and India. The successful isolation and characterization of local isolates of Mccp has provided an opportunity for the researcher to develop indigenous vaccine for the control of CCPV in Pakistan.

Acknowledgement: We are highly thankful to Pakistan Science Foundation (PSF) for funding the project PSF/NSL/P-KP-AU (219) entitle “Prevalence and Molecular Characterization of CCPV Isolates in Small Ruminants of Khyber Pakhtunkhwa.” and enabling this study possible. We would like to thank Dr. Francois Thiencourt and Manso-Silvan from CIRAD France for their technical support and guidance to conduct this study.

REFERENCES